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Molecular tagging and selection for sugar type in carrot roots using co-dominant, PCR-based markers

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Abstract

Carrot storage roots accumulate free sugars. The type of sugar accumulated is conditioned by the *Rs* locus so that typical carrot roots (*Rs*/-) accumulate predominantly glucose and fructose while *rs*/*rs* plants accumulate predominantly sucrose. We recently have found *rs*/*rs* plants in one inbred line that harbor a naturally occurring insertion sequence of 2.5 kb integrated into the first intron region of acid soluble invertase isozyme II. Using these facts, three primers were designed to differentiate *Rs*/*Rs*, *Rs*/*rs* and *rs*/*rs* carrot plants with simple PCR amplification. Co-dominant, PCR-based markers for acid soluble invertase isozyme II allowed genotyping of the *Rs* locus in 1-week-old carrot seedlings whereas mature carrot roots were needed to make this evaluation previously, and homozygous dominant plants could not be differentiated from heterozygotes without lengthy progeny testing. Marker-assisted evaluation and selection of carrot root sugar type were exercised in segregating families of diverse background and complete accuracy in predicting sugar type was realized in subsequent generations to further confirm that acid soluble invertase isozyme II conditions the *Rs* locus. These PCR-based markers will be useful in carrot breeding programs screening for this trait in segregating populations, for studying the distribution and origins of this trait in domestic and wild carrots, and for identifying seed mixtures as low as 10% *Rs*/- or 1% *rs*/*rs*.

Introduction

Carrot (*Daucus carota* L.) is a major vegetable crop worldwide with annual production around 18.5 million metric tons, annual US farm value of $500 million and annual world seed value of $90 million (Simon 2000). Carrots are an important source of pro-vitamin A, fiber and other dietary nutrients (Simon 2000), they are one of the most efficient biomass-accumulating crops (O'Hare et al. 1983; Munger 1987) and efforts to genetically improve carrot flavor, nutritional value and biomass levels are underway.

Carrots are among a few vegetable crops which accumulate free sugars (sucrose, fructose and glucose) as the predominant storage carbohydrates. The *Rs* locus conditions the type of sugars which accumulate in carrot storage roots: either predominantly sucrose in *rs*/*rs* plants or predominantly glucose and fructose (reducing sugars) in *Rs*/- plants (Freeman and Simon 1983). Free sugars are also a major component of carrot flavor

and taste panelists have found low reducing sugar (*rs*/*rs*) carrots to be less sweet and consequently less preferred than the high reducing sugar (*Rs*/-) carrots (Simon et al. 1980). In contrast, the use of low reducing sugar (high sucrose) carrots diminishes non-enzymatic browning in carrot chips and other carrot products prepared by cooking in oil at high temperature (Simon et al. 1980). We have also observed a relationship between sugar type and sugar amount, so that selection for higher total sugar content can be realized in *rs*/*rs* than *Rs*/*Rs* populations (Stommel and Simon 1989). A similar relationship between sugar type and sugar amount has been observed in tomato, where a QTL for higher sugar content was found to be an invertase gene which conditions sucrose accumulation rather than the typical reducing sugars (Fridman et al. 2000). We recently have found that the *Rs* locus of carrot inbred B4367 is associated with the acid soluble invertase isozyme II gene where isoline B4367 *rs*/*rs* harbors a 2.5 kb insertion element in this gene, while B4367 *Rs*/*Rs* has no insert in this gene (Yau and Simon 2003).

To evaluate sugar type in segregating carrot populations, a rapid DNSA (3,5-dinitrosalicylic acid) method for differentiating *rs*/*rs* roots from *Rs*/- roots has been developed (Simon and Freeman 1985) but this technique requires mature storage roots from plants at least 75 days old and this trait cannot be readily studied in wild carrots which form a fibrous, woody tap root. Furthermore, *Rs*/*Rs* and *Rs*/*rs* roots cannot be differentiated with this or any other available method since dominance of the *Rs* allele makes these two genotypes indistinguishable based upon analysis of sugar type or amount (Freeman and Simon 1983). Therefore an additional generation of progeny testing is required to make this distinction. For these reasons an efficient, simple method for unequivocally scoring the status of the *Rs* locus in carrot seedlings has immediate application for carrot breeders.

Although one AFLP marker (P3B30XA) has been found to be loosely linked to the *Rs* locus (Vivek and Simon 1999), a tightly linked, PCR-based marker for *Rs* would be useful. In this paper, we present a method using the variant region of the carrot acid soluble invertase isozyme II gene to develop three primers which can be used in a simple PCR amplification to rapidly and unambiguously differentiate *Rs*/*Rs*, *Rs*/*rs* and *rs*/*rs* carrot plants in as early as one-week-old seedlings. These primers were then used to evaluate the status of the *Rs* locus in diverse carrot populations and to predict and select the type of sugar accumulated in subsequent populations with complete accuracy.

Materials and methods

Plant materials for marker development

To develop and evaluate markers for plants of known genotype at the *Rs* locus, near-isogenic carrot lines B4367Rs (*Rs*/*Rs*) and B4367rs (*rs*/*rs*) were used (Yau and Simon 2003). Seeds were sown in soil (2 field soil:1 peat moss:1 sand:1 vermiculite) and grown in pots in the greenhouse at the University of Wisconsin-Madison, with a 14 h photoperiod at 22–25 °C. Leaves and roots of 1-week-old seedlings were harvested, rinsed, and immediately used for genomic DNA extraction.

Plant materials for marker evaluation and marker-based selection

To evaluate co-segregation between the co-dominant markers developed and the *Rs* locus (i.e. sugar type), each plant in seven F_2 populations was evaluated for both molecular marker status and sugar type. Seeds were sown either in pots in the greenhouse or in fields at Hancock, Wisconsin, and leaves were collected for marker analysis 1–5 weeks after emergence from four F_2 mapping populations heterozygous for *Rs* (B3080 × B3640, B3615 × B10138, Yc7262 × B9304, and QAL × B493); and three F_2 populations homozygous for *rs* (B493 × B3080) or *Rs* (B6274 × F524 and Brasilia × HCM) (Table 1) (Stommel and Simon 1989; Simon et al. 1990; Simon et al. 1997; Vivek and Simon 1999; Santos and Simon 2002). Plants continued to grow 15–18 weeks to typical root maturity at which time roots were harvested, rinsed, and individual storage roots were sampled for sugar analysis.

Molecular marker status was evaluated in 1–5 week old F_2 plants and this information was used to select for sugar type in F_3 progeny. Based on marker status, one to three F_2 plants each of the *rs*/*rs*, *Rs*/*Rs*, and *Rs*/*rs* genotypes were chosen from three of the four segregating F_2 populations,

Table 1. *Rs* marker status and sugar types in carrot populations. The F_3 and F_4 populations were derived from F_2 to F_3 parental plants, respectively, selected based upon their marker status.

Population	Generation	Parental status[a]		Progeny				
		Trait	Status	Observed trait segregation			Expected trait segregation	X^{2b}
				1.3 kb marker class and their sugar type	Heterozygous marker class and their sugar type	1.6 kb marker class and their sugar type		
From F_2s segregating for the *Rs* locus								
B3080 × B3640	F_2	markers	Ht	9	27	14	12.5:25:12.5	1.32
		sugars	FG	Su	FG	FG	12.5:37.5	1.31
	F_{3R}	markers	1.3	10	0	0	10:0:0	0
		sugars	Su	Su			10:0	0
	F_{3D}	markers	1.6	0	0	10	0:0:10	0
		sugars	FG			FG	0:10	0
	F_{3H}	markers	Ht	2	6	2	2.5:5:2.5	0.40
		sugars	FG	Su	FG	FG	2.5:7.5	0.13
	F_{4R}	markers	1.3	10	0	0	10:0:0	0
		sugars	Su	Su			10:0	0
	F_{4D}	markers	1.6	0	0	10	0:0:10	0
		sugars	FG			FG	0:10	0
B3615 × B10138	F_2	markers	Ht	51	124	60	58.75:117.5:58.75	1.41
		sugars	FG	Su	FG	FG	58.75:176.25	1.11
	F_{3R}	markers	1.3	10	0	0	10:0:0	0
		sugars	Su	Su			10:0	0
	F_{3D}	markers	1.6	0	0	10	0:0:10	0
		sugars	FG			FG	0:10	0
	F_{3H}	markers	Ht	4	4	2	2.5:5:2.5	1.20
		sugars	FG	Su	FG	FG	2.5:7.5	1.20
	F_{4R}	markers	1.3	10	0	0	10:0:0	0
		sugars	Su	Su			10:0	0
YC7262 × B9304	F_2	markers	Ht	9	16	6	7.75:15.5:7.75	0.61
		sugars	FG	Su	FG	FG	7.75:23.25	0.27
QAL × B493	F_2	markers	Ht	46	93	41	45:90:45	0.48
		sugars	FG	Su	FG	FG	45:135	0.03
	F_{3R}[c]	markers	1.3	10(ea)	0	0	10:0:0	0
		sugars	Su	Su			10:0	0
	F_{3D}[d]	markers	1.6	0	0	10(ea)	0:0:10	0
		sugars	FG			FG	0:10	0
	F_{3H}	markers	Ht	27	48	23	24.5:49:24.5	0.37
		sugars	FG	Su	FG	FG	24.5:73.5	0.35
	F_{3H}	markers	Ht	38	84	42	41:82:41	0.29
		sugars	FG	Su	FG	FG	41:123	0.29
	F_{3H}	markers	Ht	27	54	23	26:52:26	0.47
		sugars	FG	Su	FG	FG	26:78	0.05
Populations from F_2s not segregating for the *Rs* locus								
B493 × B3080	F_2	markers	1.3	24	0	0	24:0:0	0
		sugars	Su	Su			24:0	0
	F_{3R}	markers	1.3	10	0	0	10:0:0	0
		sugars	Su	Su			10:0	0
B6274 × F524	F_2	markers	1.6	0	0	37	0:0:37	0
		sugars	FG			FG	0:37	0
	F_{3D}	markers	1.6	0	0	10	0:0:10	0
		sugars	FG			FG	0:10	0

Table 1. (Continued)

Population	Generation	Parental status[a]		Progeny				
		Trait	Status	Observed trait segregation			Expected trait segregation	X^{2b}
				1.3 kb marker class and their sugar type	Heterozygous marker class and their sugar type	1.6 kb marker class and their sugar type		
BR × HCM	F_2	markers	1.6	0	0	43	0:0:43	0
		sugars	FG			FG	0:43	0
	F_{3D}[e]	markers	1.6	0	0	10 (ea.)	0:0:10	0
		sugars	FG			FG	0:10	0

[a]Parental marker status of 1.3, 1.6, or Ht indicates the parent plant self-pollinated to generate the progeny enumerated had the 1.3 kb band, the 1.6 kb band, or both bands, respectively; parental sugar status of Su or FG indicates that the parent plant had either predominantly sucrose with little fructose + glucose, or predominantly fructose + glucose with little sucrose, respectively.
[b]$p < 0.05$ in all cases.
[c]Two families of 10 plants each.
[d]Three families of 10 plants each.
[e]Four families of 10 plants each.

selected plants were grown for 15–18 weeks and roots were sampled for sugar analysis. Selected plants were then vernalized and self-pollinated to generate putative *rs*/*rs, Rs*/*Rs*, and *Rs*/*rs* F_3 populations. These populations were designated as F_{3R}, F_{3D}, and F_{3H}, respectively in Table 1. Using procedures described for selection in the F_2 populations, three homozygous F_3 plants were selected from segregating F_3 populations based on marker status, vernalized, and self-pollinated to continue the selection process to the F_4 generation. Putative *rs*/*rs* and *Rs*/*Rs* F_3 plants were selected and self-pollinated. Progeny were designated F_{4R} and F_{4D}, respectively. The F_{3R} and F_{4R} populations derived from *rs*/*rs* plants were expected to generate 100% high sucrose plants (designated 'Su' in Table 1) if marker-based selection was correct; the F_{3D} and F_{4D} populations derived from *Rs*/*Rs* plants were expected to generate 100% high reducing sugar plants (designated 'FG' in Table 1) if marker-based selection was correct; and the F_{3H} populations derived from *Rs*/*rs* plants were expected to generate segregating populations if marker-based selection was correct.

Sugar type could not be selected in F_2 and F_3 populations that were not segregating at the *Rs* locus, but these populations were evaluated for molecular marker status and sugars to confirm the association between these two traits. Non-segregating families that were evaluated included, B493 × B3080 (*rs*/*rs*), B6274 × F524 (*Rs*/*Rs*) and Brasilia (BR) × HCM (*Rs*/*Rs*) (Table 1).

DNA isolation

Two methods were used for genomic DNA isolation. For DNA extraction from seedlings of carrot lines B4367Rs and B4367rs, DNeasy™ Plant Mini Kit (QIAGEN Inc., Santa Clarita, CA, USA) was used. All DNA extraction procedures followed the instructions provided by the manufacturer. For DNA extraction of the F_2, F_3, and F_4 populations, genomic DNA of individual plants was isolated from freeze-dried leaves by a modified CTAB extraction method (Murray and Thompson 1980). DNA concentration and purity were measured by UV absorbance with a DU™ 640 spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA).

PCR primers and conditions

Sense primers INV-L (5′-TGA ATG CGG AGC CGC CGG CTA ATT-3′) and INV-rs (5′-GGA ATT TAA GGA TAC TTC CAA AAC-3′), and antisense primer INV-R (5′-ATT CTA CAA GGG ATG GTA CCA TTT A-3′) (see Figure 1) were used together to amplify bands to differentiate *Rs*/*Rs, Rs*/*rs* and *rs*/*rs* carrot plants. All water used was from a Milli-Q UF Plus Water System™ (Millipore Corporation, Bedford, MA, USA) and autoclaved. Takara LA *Taq*™ DNA polymerase (Panvera, Madison, Wisconsin, USA) was used for PCR amplification. Each reaction mixture

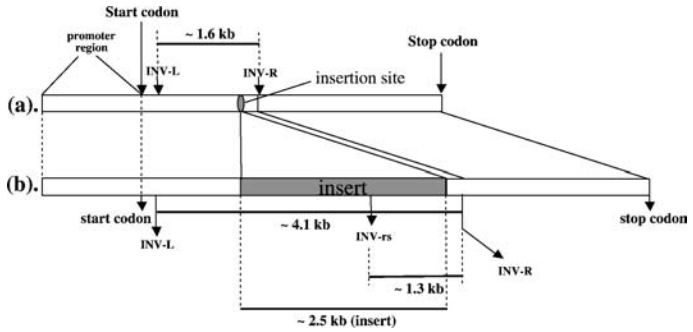

Figure 1. Genomic DNA organization of carrot acid soluble invertase isozyme II gene (a) wild type (*Rs*) and (b) mutant (*rs*) alleles. The region labeled 'insert' indicates the ~2.5 kb insert (b) (Yau and Simon, 2003). The flanking region (hatched bars) indicates the wild type carrot acid soluble invertase isozyme II gene. Three primers used to distinguish genotypes anneal 5′ (INV-L), 3′ (INV-R) and within the insert (INV-rs). The diagram is not drawn to scale.

included 9.8 μl autoclaved Milli^{TM} -Q water, 2 μl 2.5 mM dNTP mix (2.5 mM each), 2 μl 10× *LA* PCR buffer (containing Mg^{++}), 0.15 μl (5 Uμl^{-1}) *Takara LA Taq*^{TM} DNA polymerase, 2 μl of each primer (10 μM) and 2 μl 20 ngμl^{-1} DNA template. The standard PCR conditions for *Takara LA Taq*^{TM} DNA polymerase enzyme and carrot DNA concentration of 20 ng μl^{-1} were: 1 cycle at 94 °C for 3 min, 35 cycles at 94 °C (30 s), 56 °C (1 min) and 72 °C (2.5 min), then finally 1 cycle at 72 °C for 10 min.

Modified PCR cycling conditions and buffer utilized (1) *Takara* 10 × *LA*II buffer (containing Mg^{++}), which is designed for long distance PCR and (2) a modified PCR cycling program (1 cycle of 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 55 °C for 1 min, 68 °C for 3.5 min and a final cycle 72 °C for 10 min). DNA concentration of 15 ngμl^{-1} was used for PCR amplification. All PCR amplifications were carried out with a Perkin-Elmer *GeneAmp*^{TM} PCR system 9600 machine. 'Hot start' was used for PCR amplification to avoid non-specific amplicons.

Sensitivity of PCR amplification with primers INV-R, INV-L and INV-rs for Rs/Rs, Rs/rs and rs/rs plants

To determine the influence of competition between the synthesis of the two bands in *Rs*/*rs* samples during the PCR amplification, a series of artificial 'heterozygous' (*Rs* + *rs*) DNA samples were made from mixing different percentages of DNA amounts from B4367Rs (*Rs*/*Rs*) and B4367rs (*rs*/*rs*) genomic DNA. DNA mixtures of 1%:99%, 5%:95%, 10%:90%, 20%:80%, 40%:60%, 60%:40%, 80%:20%, 90%:10%, 95%:5%, 99%:1% (B4367Rs (*Rs*/*Rs*):B4367rs (*rs*/*rs*)) were evaluated.

Sugar analysis

Roots were evaluated with standard reducing sugar analytical method, which involves the extraction of juice from mature carrot storage roots and exposure of the juice to 0.5% 3,5-dinitrosalicylic acid (DNSA) (Simon and Freeman 1985). This method distinguishes between *rs*/*rs* and *Rs*/- plants.

Data analysis

Goodness-of-fit of segregation ratios was evaluated with standard χ^2 test. Estimates of the minimum family sizes for linkage analysis using in this experiment were calculated according to Hanson (1959).

Results

Marker development

Co-dominant markers were synthesized for predicting reducing sugar accumulation in carrot

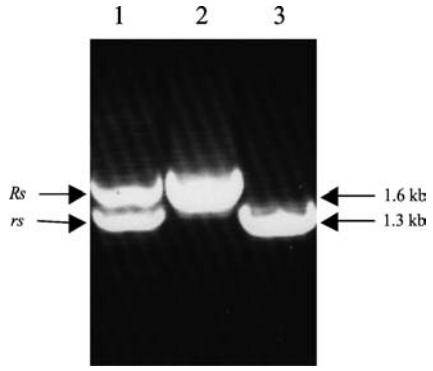

Figure 2. PCR products of *Rs*/*rs* (lane 1), *Rs*/*Rs* (lane 2) and *rs*/*rs* (lane 3) carrot plant genomic DNA amplified with a mixture of three primers INV-R, INV-L and INV-rs. Standard PCR conditions were used for amplification.

roots. Based on the fact that a 2.5 kb insert is integrated into the acid soluble invertase isozyme II gene of B4367rs (*rs*/*rs*) but not in the same gene of B4367Rs (*Rs*/*Rs*) carrot plants (Figure 1; Yau and Simon 2003), three primers INV-L, INV-R and INV-rs were developed for PCR to differentiate *Rs*/*Rs*, *Rs*/*rs* (both of which predominantly accumulate reducing sugars glucose and fructose) and *rs*/*rs* (storage roots predominantly accumulate disaccharide sucrose) carrots using genomic DNA. Primer INV-rs targeted the insertion region and primers INV-R and INV-L targeted the acid soluble invertase isozyme II gene flanking the insert (Figure 1).

Two bands of approximately 1.3 kb and 1.6 kb were amplified when primers INV-R, INV-L and INV-rs were used together in a polymerase chain reaction with genomic DNA of a heterozygous *Rs*/*rs* plant (Figure 2 lane 1). A 1.3 kb band Figure 2 lane 3) was amplified from the genomic DNA of carrot B4367rs (*rs*/*rs)* while a 1.6 kb band was synthesized from the genomic DNA of B4367Rs (*Rs*/*Rs*) (Figure 2 lane 2). Sequencing of the 1.3 kb DNA fragment yielded the DNA sequence of the 3′ end of the insertion and adjacent acid soluble invertase isozyme II circumscribed by sense primer INV-rs and antisense primer INV-R (Figure 1), as expected. The sequence of the 1.6 kb band from *Rs*/*Rs* plants was identical to that published by Unger et al. (1992), as expected.

To confirm the primer specificity for *Rs* and *rs* alleles, further PCR study was performed. The combination of primers INV-R and INV-L generated a single band (~1.6 kb) with DNA from *Rs*/*Rs* plants (B4367Rs), or with a mixture of B4367Rs + B4367rs DNA, while no bands were observed from *rs*/*rs* plants (B4367rs) (data not presented), so this primer pair was specific for the *Rs* allele. The combination of primers INV-R and INV-rs generated no PCR product from genomic DNA of *Rs*/*Rs* carrot plants while a ~1.3 kb band was generated with DNA from *rs*/*rs* plants (B4367rs), or with a mixture of B4367Rs + B4367rs DNA, so this primer pair was specific for the *rs* allele. The combination of primers INV-L and INV-rs generated no bands in any DNA samples, whereas the combination of all three primers generated a 1.6 kb band with B4367Rs DNA, a 1.3 kb band with B4367rs DNA, and both bands with either a mixture of B4367Rs and B4367rs DNA or DNA from a heterozygous plant, as expected.

Although a 4.1 kb product might be predicted, no PCR product was generated from B4367rs DNA amplified with INV-R and INV-L using our standard PCR conditions and 10x *LA* buffer. However, a 4.1 kb PCR product was produced with *rs*/*rs* genomic DNA and primers INV-R + INV-L using modified PCR cycling conditions and buffer described in Materials and Methods (data not presented). Using all 3 primers and the modified PCR conditions, two bands (~4.1 kb and ~1.3 kb) were produced although only a faint band of ~4.1 kb was amplified, presumably due to the competition in the synthesis of these two bands.

Sensitivity of PCR amplification with primers INV-R, INV-L and INV-rs for Rs/*Rs, Rs*/*rs and rs*/*rs plants*

To determine competition in the synthesis of the 1.3 kb and 1.6 kb bands in *Rs* + *rs*, mixtures of synthetic 'heterozygous' (*Rs*/*rs*) DNA samples were made by mixing varying percentages B4367Rs (*Rs*/*Rs*) and B4367rs (*rs*/*rs*) genomic DNA and used for PCR amplification (Figure 3). Both bands could be clearly observed from a sample with a 20% *Rs*:80% *rs* mixture (Figure 3), while a weak 1.6kb band (marker for the *Rs* allele) was synthesized when mixtures ranging from 10% *Rs*:90% *rs* to 99% *Rs*:1% *rs* were used for PCR amplification. No *Rs* allele marker (1.6 kb band)

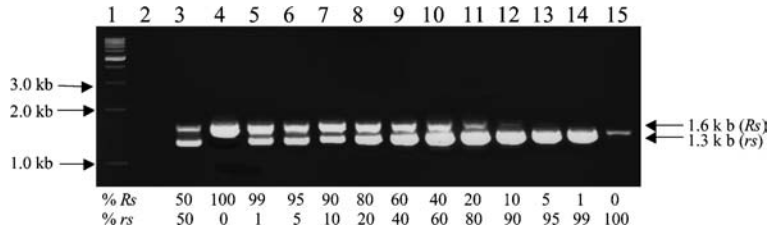

Figure 3. Products of standard PCR cycling conditions using *Rs* + *rs* DNA mixtures of B4367Rs (*Rs*/*Rs*) and B4367rs (*rs*/*rs*) genomic DNA as templates and amplified with primers INV-R, INV-L and INV-rs. Lanes 1:1 kb DNA size markers; lane 2: negative control (PCR without DNA); lane 3: positive control (heterozygous *Rs*/*rs* plant); lane 4: B4367Rs (100%) + B4367rs (0%); lane 5: B4367Rs (99%) + B4367rs (1%); lane 6: B4367Rs (95%) + B4367rs (5%); lane 7: B4367Rs (90%) + B4367rs (10%); lane 8: B4367Rs (80%) + B4367rs (20%); lane 9: B4367Rs (60%) + B4367rs (40%); lane 10: B4367Rs (40%) + B4367rs (60%); lane 11: B4367Rs (20%) + B4367rs (80%); lane 12: B4367Rs (10%) + B4367rs (90%); lane 13: B4367Rs (5%) + B4367rs (95%); lane 14: B4367Rs (1%) + B4367rs (99%); lane 15: B4367Rs (0%) + B4367rs (100%).

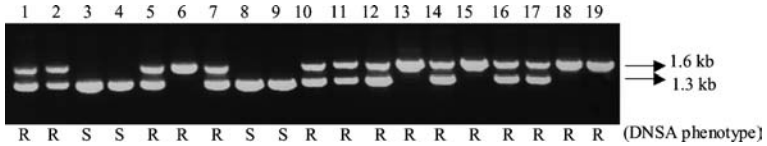

Figure 4. PCR products of carrot genomic DNA from an F_2 mapping population, Yc7262 × B9304, using standard PCR cycling conditions with primers INV-R, INV-L and INV-rs. DNSA test results are indicated with 'R' and 'S'. 'R' indicates that the major sugar types in carrot storage root were reducing sugars (fructose and glucose). 'S' indicates that the major sugar type in carrot mature root was sucrose.

was observed in the mixtures of 5% *Rs*:95% *rs* (Figure 3 lane 13). In contrast, both co-dominant markers (1.6 and 1.3 kb) were generated in a mixture of 99% *Rs*:1% *rs* indicating that the shorter product (from *rs* allele) was preferentially synthesized even with abundant (99%) *Rs* allele (Figure 3 lane 5). As expected, the competition effect was eliminated by amplifying *Rs* and *rs* alleles separately with primers INV-R and INV-L (for *Rs)*, and INV-rs and INV-R (for *rs*) and the expected band was amplified in mixtures with as little of 0.5% of the total DNA (data not presented).

PCR amplification, sugar type, and marker-assisted selection in F_2, F_3 and F_4 populations

F_2 families segregating for the *Rs* locus were developed, based on previous evaluation of parental inbreds found to be true-breeding for sugar type (Freeman and Simon 1983; Stommel and Simon 1989). Individual carrot plants from four F_2 populations segregating for the *Rs* locus were scored for PCR marker status in 1–5 week seedlings and sugar type was evaluated in mature roots with DNSA. All F_2 roots with either both marker bands (Figure 4 lanes 1, 2, 5, 7, 10, 11, 12, 14, 16 and 17) or the 1.6 kb marker band Figure 4 lanes 6, 13, 15, 18 and 19) had storage roots with high reducing sugar accumulation (Table 1), as expected for *Rs*/*rs* or *Rs*/*Rs* plants. All plants with only the 1.3 kb band had storage roots with high sucrose (low reducing sugar levels) based on the DNSA test, confirming that they were *rs*/*rs* (Figure 4 lanes 3, 4, 8 and 9). Five putative *Rs*/*rs* F_2 plants from three families, based on PCR results, were self-pollinated and all yielded F_3 populations segregating for both markers and sugar type and designated as F_{3H} in Table 1. A total of 496 F_2 plants in four segregating families and 386 F_3 plants in five segregating families were scored for molecular marker status and sugar type. In all cases molecular marker status predicted sugar type, i.e. progeny with the 1.6 kb band only, or with both bands, always had storage roots with predominantly reducing sugars (*Rs*/*Rs* and *Rs*/*rs* progeny, respectively); while all progeny with the 1.3 kb band always had storage roots with predominantly sucrose (*rs*/*rs* progeny). Trait

segregation fit expected 1:2:1 or 3:1 ratios for all families.

From the segregating F_2 families, four F_2 plants that generated the 1.3 kb marker only were selected among 5 week-old seedlings, grown to maturity, and self-pollinated to generate F_{3R} families and five plants which generated the 1.6 kb marker only were selected and self-pollinated to generate F_{3D} families. From the segregating F_3 families, two F_3 plants which generated the 1.3 kb marker were self-pollinated to generate F_{4R} families and one family categorized as *Rs*/*Rs* was self-pollinated to generate a F_{4D} family. All of these families derived from plants homozygous for markers were true-breeding based upon DNSA and marker evaluation of 10 plants per family (Table 1).

Based upon previous evaluation of parental stocks for sugar type, three F_2 families not segregating for the *Rs* locus were developed and evaluated (Table 1, bottom). Two *Rs*/*Rs* F_2 families uniformly generated the 1.6 kb marker only, as expected for the *Rs*/*Rs* genotype, and based on our observations of high reducing sugar content with DNSA results among 37 or 43 plants evaluated. Five randomly selected plants were self-pollinated and all F_{3D} progeny plants generated only the 1.6 kb marker and high reducing sugar storage roots among 10 plants evaluated per family, as expected for *Rs*/*Rs* plants. One *rs*/*rs* F_2 family generated the 1.3 kb marker with PCR analysis and high sucrose storage roots among 24 plants, and the F_{3R} family derived from this family generated only the 1.3 kb marker with PCR analysis and high sucrose storage roots expected for the *rs*/*rs* genotype among 10 plants evaluated.

In summary, a total of 1176 plants from 7 F_2 families derived from 12 diverse carrot inbreds and their selected F_3 and F_4 progeny were evaluated for PCR amplification products developed to evaluate *Rs* and *rs* alleles, and for predominant sugar stored in mature storage roots. In all cases, plants scored as *rs*/*rs* at the seedling stage developed storage roots which stored predominantly sucrose while *Rs*/*rs* and *Rs*/*Rs* plants all stored predominantly reducing sugars (glucose + fructose). Marker-assisted selection was successful in predicting the type of sugar stored in 11 F_3 families and 3 F_4 families. The markers and sugar type fit expected 1:2:1 or 3:1 segregation ratios in all cases.

Discussion

Invertases irreversibly catalyze the breakdown of the disaccharide, sucrose, into fructose and glucose and are present in most plant tissues in multiple forms. Acid invertases are divided into soluble (vacuolar) and insoluble (extracellular or cell wall-bound) isoforms. Carrot contains both acid soluble and insoluble invertases (Unger et al. 1994; Lee and Sturm 1996). It has been suggested that acid invertases are involved in phloem unloading (Eschrich 1980), control of sugar type in storage organs (Lauriere et al. 1988), normal development of endosperm (Cheng et al. 1996), wound response (Sturm and Chrispeels 1990; Zhang et al. 1996) and response to pathogen infection (Sturm and Chrispeels 1990). We have also found that the acid soluble invertase isozyme II gene is the candidate for the *Rs* locus in inbred B4367, and the low invertase activity in *rs*/*rs* roots is associated with the incorporation of an insertion sequence into this gene (Yau and Simon 2003).

The acid soluble invertase isozyme II gene plays an important role in carbohydrate metabolism, especially during carrot root development (Sturm et al. 1995; Sturm 1996). In our earlier study using near-isogenic lines B4367Rs (*Rs*/*Rs*) and B4367rs (*rs*/*rs*) and northern analysis we found that no wild type acid soluble invertase isozyme II mRNA was detected during the development of B4367rs carrot roots, which accumulate sucrose, while the transcript was detected in B4367Rs roots, which accumulate glucose and fructose (Yau and Simon 2003).

Genetic variation in invertase isozymes has also been studied in tomato where sucrose accumulation is a monogenic recessive trait (*sucr*) correlated with reduced invertase transcript (Stommel and Haynes 1993). Comparing *Lycopersicon* species, invertase transcription increased throughout fruit development in *L. esculentum* and *L. pimpinellifolium* fruit (which do not accumulate sucrose) while *L. chmielewskii* accumulates sucrose in its fruits, and no recognizable *sucr* transcript was observed (Klann et al. 1993). These studies provided strong evidence that, as in carrot roots, the activity of invertase directly influences the partitioning of sucrose, fructose and glucose in the fruit of ripening tomato.

In this study, we have successfully developed and used three primers (INV-R, INV-L and INV-rs) to generate co-dominant markers to

differentiate *Rs*/*Rs*, *Rs*/*rs* and *rs*/*rs* carrot plants. This co-dominant, PCR-based, genomic DNA system provides a powerful tool for carrot breeders to score the genotype of plants for the *Rs* locus in carrot seedlings as young as one week old. Seed mixtures as low as 1% *rs*/*rs* or 10% *Rs*/*Rs* can be detected with mixed primers for both alleles and with greater sensitivity if primers for only one allele are used.

These primers detected allelic differences of the structural locus which apparently conditions *Rs*, and co-segregation of the markers and the locus was 100% among plants in F_2 and F_3 families. Complete co-segregation of the high reducing sugar storage root phenotype, based on DNSA scores, with both the *Rs*/*Rs* (1.6 kb marker band) and *Rs*/*rs* (1.6 kb marker band + 1.3 kb marker band) PCR results was observed; while all plants which stored high sucrose content based on DNSA scores, produced the 1.3 kb marker band, as expected for *rs*/*rs* plants. This provides confidence that the insertion sequence we found in the acid soluble invertase isozyme II gene, in fact, conditions the *Rs*/*rs* trait within a margin of error of 0.04 cM around the *Rs* locus, with the number of plants evaluated (Hanson 1959). Further analysis of this region of the genome will confirm or refute our contention that a ~2.5 kb insertion sequence is, in fact, the molecular basis for variation in carrot storage root sugars conditioned by the *Rs* locus (Yau and Simon 2003). There is a possibility that some other genetic modification within the 0.04 cM region circumscribed by segregation analysis to date may, in fact, condition *Rs*. More extensive sequence analysis of this region in B4367Rs and B4367rs may provide evidence of other genetic modifications which could reside close to this invertase gene. Alternatively, use of the cloned acid soluble invertase isozyme II gene from *Rs* stocks in transgenic studies with *rs* plants would confirm the ability for this enzyme to alter the *rs* phenotype.

One difficulty encountered in evaluating carrot germplasm for storage root traits is the small, fibrous root system that develops in wild carrot. Type of sugar stored, for example, often cannot be reliably measured since the woody nature of these roots complicates extraction. Preliminary studies are underway to evaluate all publicly released carrot inbreds and populations, breeding stocks from seed companies, and a diverse collection of carrot germplasm in the USDA collection. Analysis includes both evaluation of sugar type and marker status using techniques described herein. The markers we developed provide an unequivocal evaluation of the *Rs* locus in nearly all of those plant materials, since marker status and sugar type coincided. In a few cases (< 1% thus far) we found marker products larger or smaller than that of the *rs* allele, so we conclude there are other *rs* alleles. Point mutations in key regions of the wild type allele could also result in the *rs* phenotype, and these would not be identifiable as mutations using the methods described. Further studies of the *Rs* locus in diverse carrot germplasm will be of particular interest in helping us understand the origins and natural distribution of this interesting mutation in wild and domesticated carrot populations.

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